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Cell Cycle- and Age-Dependent Transcriptional Regulation of Human Thymidine Kinase Gene: The Role of NF-Y in the CBP/tk Binding Complex

Key Words
Diploid fibroblasts, human
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NF-Y

Abstract
Expression of thymidine kinase gene in normal human diploid cells is both cell cycle- and age-dependent and appears to be transcriptionally regulated. Strong DNA protein binding with a 28-bp fragment (–91/–64) that contains the distal inverted CCAAT box is observed in serum-stimulated young (low population doubling level) IMR-90 cells but not in senescent cells. This cell cycle- and age-dependent binding factor was termed CBP/tk, indicating CCAAT binding protein for the thymidine kinase gene. Based on immunoshift assay and purification, it has been suggested that CBP/tk is equivalent to NF-Y, previously identified as the binding protein for the Y box within Eα gene promoter. In this study, we examined the mRNA level and protein amount of NF-Y proteins in young and old IMR-90 cells during serum stimulation by Northern and Western blot blot analyses. In addition, we compared (1) the turnover rate of NF-Y in IMR-90 cells with that of CBP/tk binding activity and (2) the levels of NF-Y and CBP/tk in normal and cancer cells. Both NF-YA and NF-YB were constitutively expressed at mRNA level in IMR-90 cells. However, expression of NF-YA, and to a lesser degree, NF-YB, at the protein level were clearly age-dependent. The half-life of NF-YA and NF-YB were, respectively, 4- and >10-fold longer than that of CBP/tk binding activity in IMR-90 cells. In addition, we found that the amount of NF-Y did not correlate with the overexpression of CBP/tk binding activity in HeLa cells. Taken together, our results suggested that although CBP/tk may contain NF-YA or related proteins, NF-A and NF-YB alone may not account for all the characteristics of CBP/tk observed in normal and transformed human cells.

Introduction

Thymidine kinase (TK) activity is tightly coupled to DNA synthesis in normal human diploid cells, increasing dramatically at the G1/S boundary of the cell cycle [1]. Sequence-specific binding to a 28-bp DNA fragment containing one of the two inverted CCAAT boxes in the promoter is cell cycle- and age-dependent and the binding factor was termed

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as CBP/tk, indicating CCAAT binding protein for the TK gene [2]. The Yi-like and E2F-like sequences within the TK promoter do not contribute to the cell cycle- and age-dependent CBP/tk binding [3].

NF-Y was originally described as a *trans*-acting factor recognizing the Y box of major histocompatibility complex (MHC) class II genes [4]. NF-Y is either identical or homologous to CCAAT binding proteins variously called CP1, which binds to a CCAAT sequence in the adenovirus major late promoter (MLP) [5], CBF [6], and HAP in yeast [4]. These proteins recognize CCAAT boxes in a large number of other genes including herpes simplex virus TK, mouse MHC class I, mouse albumin, and α- and β-globin [4, 5]. Purification of CBP/tk binding activity in human HL-60 cells using the 28-bp DNA affinity chromatography has identified NF-YA and NF-YB as two components responsible for CCAAT binding activity [8]. However, comparison of several biochemical properties shows differences between CBP/tk and NF-Y [2]. Thus, although competition and immunoshift analysis suggest that NF-Y- or NF-Y-like proteins may be involved in CBP/tk binding, it is not certain that CBP/tk is identical to NF-Y. CBP/tk, in addition to being a CCAAT binding protein, has some unique features including: (1) its binding is cell cycle-dependent; (2) its binding is age-dependent; (3) it has a very short half-life, less than 60 min, and (4) it is overexpressed in cancer cells [2, 9]. In the present study, these features are used to further assess the role of NF-Y in the CBP/tk binding complex.

Methods

Materials and Cell Culture

All tissue culture media and sera were obtained from GIBCO, Grand Island, N.Y., USA. [γ-^{32}P]ATP (3,000 Ci/mmol) was from ICN Chemical Radioisotope Division, Irvine, Calif., USA. Restriction enzymes and other molecular biological supplies were from Promega, Madison, Wisc., USA, or Pharmacia, Piscataway, N.J., USA. All other chemicals were of reagent grade. IMR-90 human embryonic lung fibroblasts were obtained from the Coriell Institute for Medical Research, Camden, N.J., USA.

Oligonucleotides

The following oligonucleotides: the 28-bp fragment (28-bp, 5′-dAGGTCAGCGGCCGGGCGCTGATTGGCCC-3′), MLP (5′-dGATCCTTCGGCATCAAGGAAGGTGATTGGTTT), MHC class II Eα gene Y box (5′-ATTTTTCTGATTGGTTAAAAGT) were synthesized using a Pharmacia LKB Gene Assembler Plus DNA synthesizer. Complementary synthetic oligonucleotides were annealed, end labeled with [γ-^{32}P]ATP and T4 kinase, and used for the gel mobility shift assay.

Northern Blot Analysis

Total RNA was prepared for Northern blot analysis as previously described [1].

Western Blot Analysis

Whole cell lysates were prepared and fractionated by SDS-PAGE. Proteins separated within the gel were transferred to a nitrocellulose membrane for immunoblotting using various antibodies. Antisera were used at 1,000-fold dilution. The ECL immunodetection kit (Amersham, Arlington Heights, Ill., USA) was used for the detection.

Gel Mobility Shift Assays

Nuclear extracts for DNA binding were prepared as described before [2]. The gel mobility shift assay was carried out on a 4% polyacrylamide gel under conditions as previously described [2]. For immunoshift assay, 1 μl of the undiluted antiserum was added to the nuclear extracts in the binding mixture of 20 μl and incubated on ice for 20 min before the addition of probe.

Results

Table 1 shows the sequence comparison of the 28-bp human TK promoter fragment (–91 to –64) with CCAAT sequence in the adenovirus MLP and the Y box of MHC class II Eα gene (Eα 22-mer). Figure 1 shows that Eα, but not MLP, could compete effectively with the

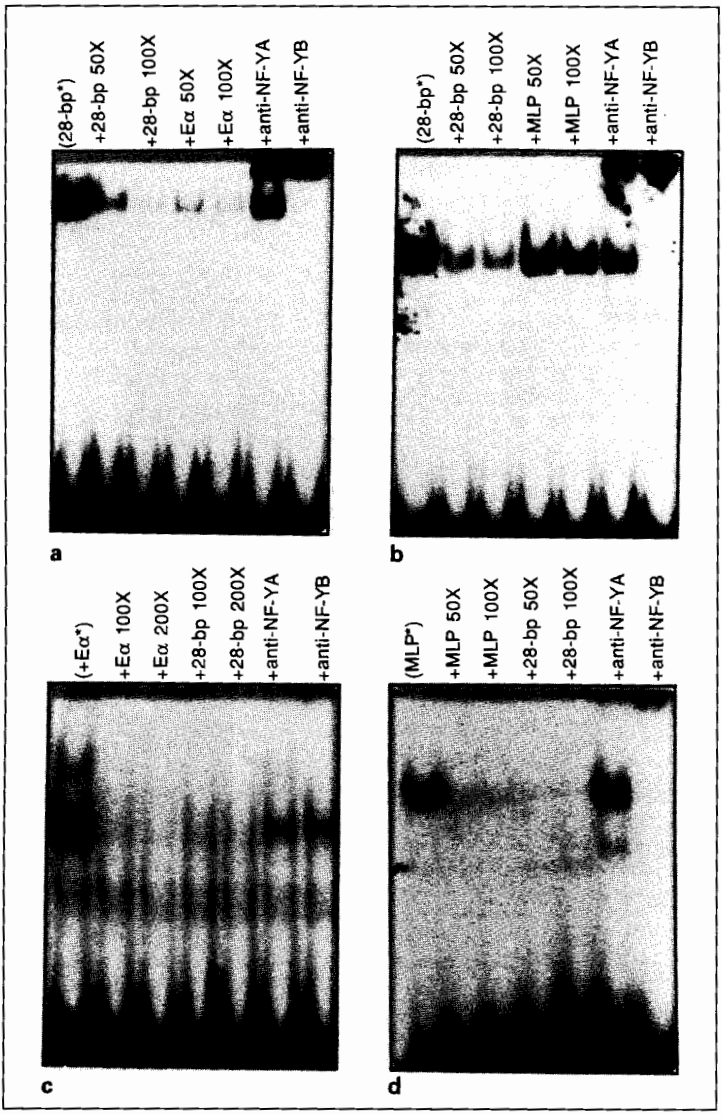


Fig. 1. Competition and immunoshift analysis. **a** The 28-bp binding (28-bp*) was competed by itself or Eα fragment at 50 and 100× concentration. **b** The 28-bp binding was competed by itself or by MLP fragment at 50 and 100× concent ration. **c** The Eα binding was competed by the itself or by the 28-bp fragment. **d** The MLP binding was competed by itself or by the 28-bp fragment. In each case, immunoshift assay was carried out with anti-NF-YA and anti-NF-YB antiserum. Each binding assay contained 10 μg of nuclear extract.

Table 1. Sequence comparison for the 28-bp TK promoter fragment and Y box containing MLP and MHC class II Eα gene promoter

28-bp	AGGTC AGCGG CCGGG CGCTG **ATTGG** CCC
23-bp	AGCGG CCGGG CGCTG **ATTGG** CCC
Eα	TTA TTCTG **ATTGG** TTAAA AGT
MLP	CTTCG GCATC AAGGA AGGTG **ATTGG** TTT

The 14-base authentic Y box is underlined. The inverted CCAAT box is in bold print.

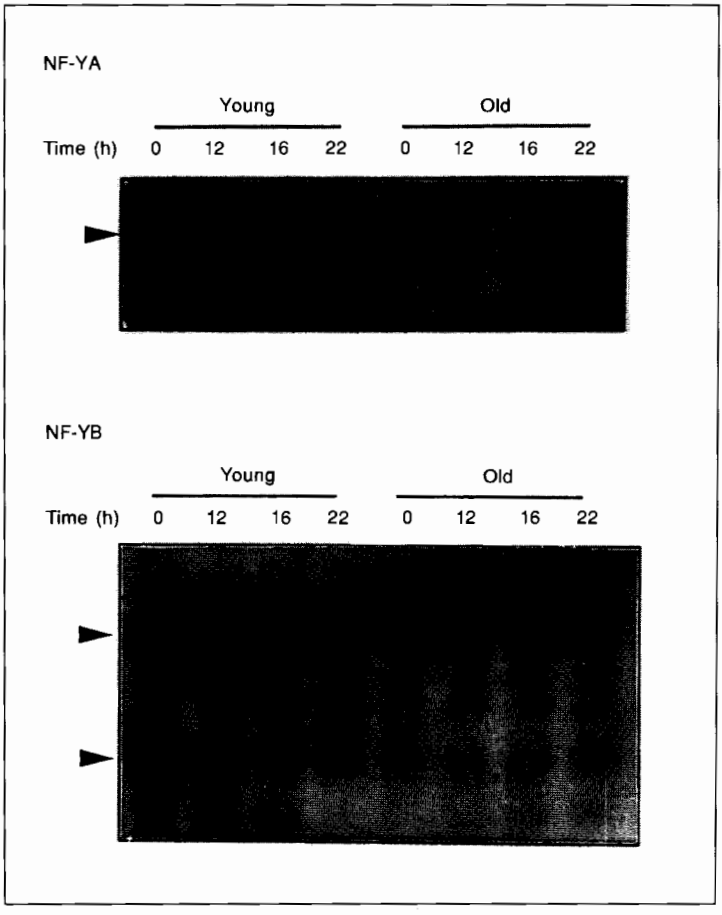
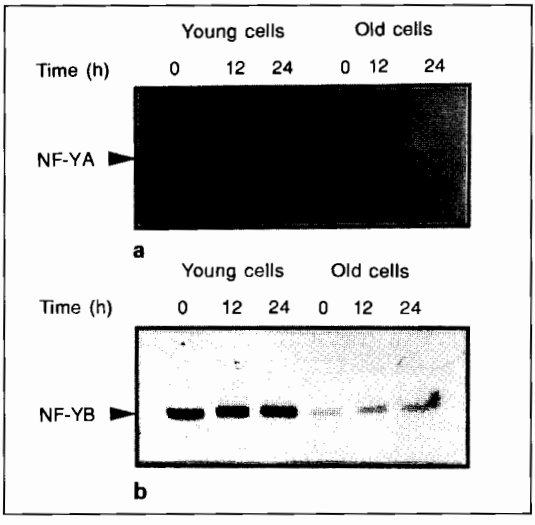


Fig. 2. Northern blot analysis of NF-YA and NF-YB in young (population doubling level = 19) and old (population doubling level = 50) cells. Confluent cultures of young and old cells were serum-deprived for 48 h and then stimulated with serum for various times as indicated. Arrows indicate the positions of NF-Y mRNA (~ 4.0 kb) and NF-YB mRNA (~ 3.0 and 1.2 kb).



28-bp fragment in the binding assay (fig. 1a, b). By contrast, the 28-bp fragment could displace both Eα and MLP in DNA binding (fig. 1c, d). In all cases, antibodies against NF-YA and NF-YB caused either a decrease in binding intensity (Eα binding) or a supershift (28-bp and MLP). A deletion from the 5′ end of the 28-bp fragment up to 5 base

Fig. 3. Western blot analysis of NF-YA (**a**) and NF-YB (**b**) in young (population doubling level = 19) and old (population doubling level = 50) IMR-90 cells. Cells were serum-deprived and then stimulated for various times as indicated. Polyclonal antisera were used at 1:500 dilution.

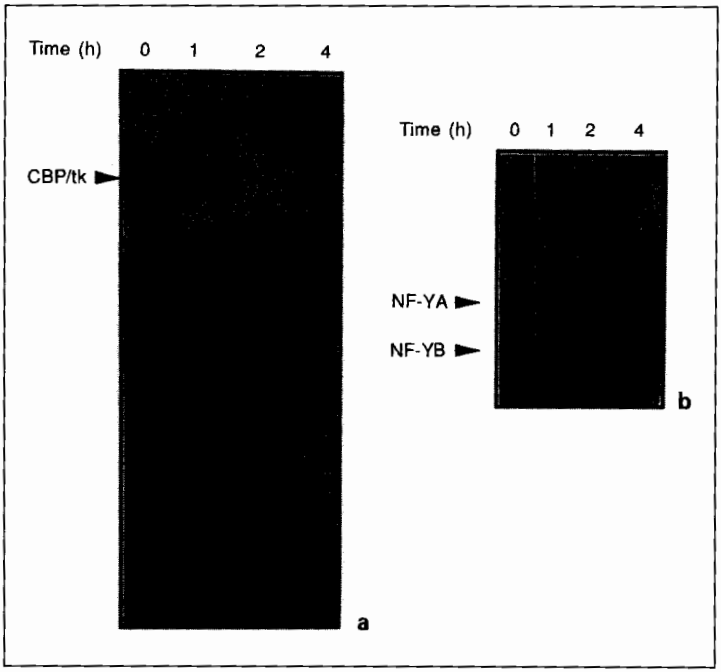


Fig. 4. Comparison of the turnover rate of CBP/tk (**a**) and NF-Y proteins (**b**) in serum-stimulated IMR-90 cells following cycloheximide treatment. IMR-90 cells (population doubling level = 19) were serum stimulated for 20 h, then treated with cycloheximide (50 μg/ml) for various times as indicated. Nuclear extracts were prepared for gel mobility shift assay using 28 bp as probe (**a**) and Western blot analysis for NF-YA and NF-YB.

pairs was sufficient to render the resulting oligonucleotide (23-bp) completely inactive in competing with the 28-bp fragment for CBP/tk binding [3], suggesting that the 5′-flanking region in the 28-bp fragment is required for CBP/tk binding activity. Taken together, the results suggest that although NF-Y or related proteins are involved in the binding of all these oligonucleotides, the precise composition of each binding complex may vary.

Figure 2 shows the expression of NF-YA and NF-YB gene in young and old IMR-90 cells after serum stimulation. Both NF-YA (~4.0 kb) and NF-YB mRNA (~3.0 and ~1.2 kb) were constitutively expressed under serum-deprived (0 h) and serum-stimulated conditions. The levels of NF-YB mRNA in young and old cells were comparable. The level of NF-YA in young cells was slightly higher (~2-fold) than in old cells. Figure 3 shows that serum stimulation caused a 3- to 4-fold increase in NF-YA protein in young cells. By contrast, NF-YA was barely detectable in old cells even after serum stimulation. NF-YB, existing as a doublet of 32.5- and 30-kDa proteins, was not serum-responsive in young or old cells. The level of NF-YB in young cells was about 2- to 3-fold higher than in old cells.

Figure 4 shows that half-life of CBP/tk binding activity in young IMR-90 cells after protein synthesis inhibition was less than 60 min, as previously reported [2]. In contrast, the half-lives of NF-YA and NF-YB were much longer, estimated to be, respectively, 180 min and >4 h. Thus, although no CBP/tk binding activity could be detected 2 h after cycloheximide treatment, the amount of NF-YA and B in the nuclear extract did not show any significant decrease.

Figure 5 compares the CBP/tk binding activity in normal IMR-90 cells and in HeLa cells. As reported previously, the level of

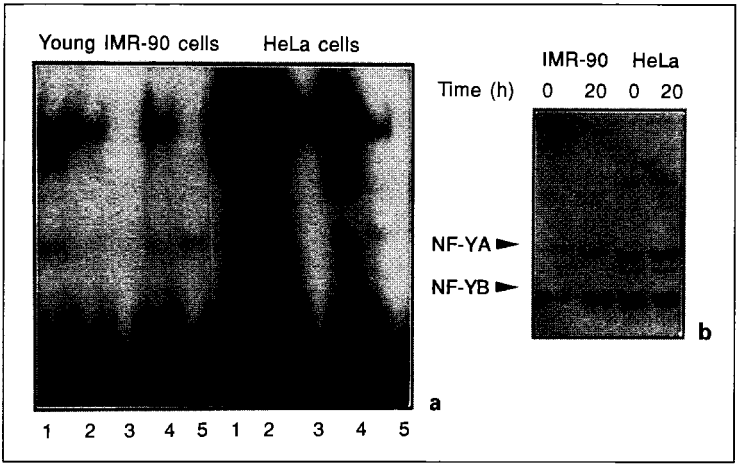


Fig. 5. CBP/tk binding activity and NF-Y proteins in young IMR-90 cells and in proliferating HeLa cells. **a** CBP/tk binding activity in IMR-90 cells and HeLa cells. Nuclear extracts from IMR-90 and HeLa cells were pretreated under different conditions: lane 1 = control; lane 2 = 37°C for 5 min; lane 3 = 65°C for 5 min; lane 4 = 10 m*M* dithiothreitol; lane 5 = 10 m*M* N-ethyl maleimide. **b** Western blot analysis of NF-YA and NF-YB in IMR-90 cells (population doubling level = 19) and HeLa cells.

CBP/tk binding in HeLa cells was about 15-fold higher than that in IMR-90 cells under the same binding conditions. However, the level of either NF-YA or NF-YB in HeLa cells was only about 3-fold higher than that in serum-stimulated IMR-90 cells.

Discussion

Many eukaryotic promoters possess one or a few *cis*-elements that contain the pentanucleotide sequence CCAAT between 60 and 120 bp upstream of the initiation site either on the coding or on the noncoding strand [10, 11]. Although these elements share the same CCAAT core sequence, their flanking sequences provide extra fine-tuning which determines the binding heterogeneity of different binding proteins. The CCAAT binding protein that recognizes the 28-bp segment within the human TK promoter was termed CBP/tk [2]. Although the 28-bp fragment and Eα 22-mer compete with each other effectively (fig. 1), the 5′-flanking sequence of the 28-bp fragment, not shared by Eα, appears to be important for CBP/tk binding.

NF-Y was originally described as a heterodimeric protein, containing NF-YA and NF-YB, that recognizes Y box and related CCAAT sequences [4]. NF-Y complex is generally considered as a constitutive activator, the same as Sp1. This seems to be the case for NF-Y at mRNA levels in IMR-90 cells (fig. 2). However, the expression of NF-YA protein, at least in IMR-90 cells, is clearly age-dependent (fig. 3). This is interesting because it would imply that many NF-Y-controlled genes may exhibit age-dependent characteristics. Although competition and immunoshift analysis (fig. 1) support the notion that NF-Y proteins are involved in CBP/tk binding, we noted that the NF-YA and NF-YB did not correlate with the CBP/tk binding activity in several regards: (1) the half-life of NF-YA/B is much longer than that of CBP/tk in IMR-90 cells (fig. 4); (2) the effect of serum is more pronounced with CBP/tk (>10-fold induction) [2] than with NF-YA (~2- to 3-fold induction) in IMR-90 cells (fig. 3); (3) NF-YB is not serum-responsive in IMR-90 cells (fig. 3), and (4) the increase in NF-Y does not correlate with the increase in CBP/tk binding activity in HeLa cells (fig. 5). These discrep-

ancies could be due to the presence of factors other than NF-YA and NF-YB in CBP/tk binding complex. Recently a third component of NF-Y, CBF-C (NF-YC), has been isolated and cloned [12]. It remains to be seen whether NF-YC or other unidentified factors may be responsible for these unique features of CBP/tk.

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